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Transepithelial fluctuation analysis of chloride secretion

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Abstract

Transepithelial fluctuation analysis (noise analysis) provides valuable information about the density and single-channel properties of ion channels in intact epithelia. Here we investigate cystic fibrosis transmembrane conductance regulator (CFTR)-dependent chloride (Cl^-) secretion in T84 human colonic epithelia by inducing noise using the diarylsulfonylurea DASU-01, a low-affinity open-channel blocker of CFTR. Our data indicate that the apical membrane of maximally stimulated T84 epithelia has a very high Cl^- conductance generated by ~ 7000 active CFTR channels per cell with open probability (P_o) of ~ 0.4 and single-channel amplitude (i) of ~ 0.1 pA. Similar experiments might provide important information about how drugs regulate CFTR in intact epithelia.

Keywords: Noise analysis; CFTR; Chloride ion channel; Channel block; Epithelial ion transport; T84 cells

1. Introduction

Transepithelial fluctuation analysis (noise analysis) is a noninvasive method whereby one can obtain estimates of the single-channel amplitude (i), channel density (N) and open probability (P_o) of an ion channel in an intact epithelium. Noise analysis has been an invaluable approach in understanding the regulation of the apical membrane epithelial sodium (Na^+) channel (ENaC). Because the spontaneous gating of ENaC is too slow, blocker-induced noise using low-affinity ENaC blockers has been used to obtain adequate amplitude of current power in a measurable frequency range 0.1–1000 Hz. The use of low-affinity blockers that cause only a small decrease in the short-circuit current (I_{SC}) also minimizes the potential influence autoregulatory mechanisms may have on the channel properties, something that has proved to be important when studying ENaC. Noise analysis has been used in patch-clamp studies to investigate the ATP-dependent gating of cystic fibrosis transmembrane conductance regulator (CFTR) in multi-channel patches [1]. Results from these studies revealed an ATP-dependent gating of CFTR in the 0.5–2-Hz range. Based on these observations, one might have expected to observe the corresponding Lorentizans in the transepithelial current power density spectra (PDS) of stimulated chloride (Cl^-) secretory cells such as T84 cell monolayers. However, this was not the case. Instead, as with ENaC, it has proven necessary to use blocker-induced noise to study CFTR in an intact epithelium. Fortunately, Sheppard and Welsh [2] had shown that the sulfonylureas such as glibenclamide and tolbutamide block CFTR. Subsequent patch-clamp studies using noise analysis on multi-channel patches [3,4] as well as event duration analysis on single-channel patches [5] have shown the sulfonylureas act as pseudo-first-order open-channel blockers of CFTR. Based on these results, we prepared a series of sulfonylurea derivatives in an effort to obtain a compound suitable for transepithelial noise analysis studies of CFTR. The compound shown in Fig. 1 (DASU-01) has proved to be of use for this purpose. Patch-clamp studies have verified DASU-01 is a low-affinity open-channel blocker of CFTR (Fig. 2). Below, we describe the protocol that we have developed to study CFTR using transepithelial noise analysis with DASU-01.

Abbreviations: 1-EBIO, 1-ethyl-2-benzimidazolone; FBS, fetal bovine serum; i, Single-channel amplitude; FSK, forskolin; I_{SC}, short-circuit current; N, channel density; ENaC, epithelial Na^+ channel; P_o, open probability; PDS, power density spectra; PBS, phosphate-buffered saline; Pen-Strep, penicillin–streptomycin.

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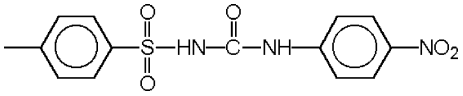

Fig. 1. Chemical structure of DASU-01.

Sample results will also be presented as well as the quantitative methods used to obtain estimates of the CFTR channel properties (i, N and P_o).

2. Materials

The following materials are required to culture T84 cells and perform transepithelial noise studies: T84 cells (American Tissue Culture Collection, cat. no. CC1-248); DMEM (Invitrogen, Carlsbad, CA, cat. no. 12100-046); F-12 (Life Technologies, cat. no. 21700-075); fetal bovine serum (FBS; Hyclone, Logan, UT, cat. no. SH30070.03); penicillin–streptomycin, Pen-Strep (Life Technologies, cat. no. 15140-122); Trypsin (Life Technologies, cat. no. 15400-054); phosphate-buffered saline (PBS; Sigma, St. Louis, MI, cat. no. P-4417); T_{25} cm^2 flasks (Fisher, Hampton, NH, cat. no. 10-126-10); transwell filters (Costar, Acton, MA, cat. no. 3470); Ussing chamber Snapwell vertical diffusion chambers (Harvard Apparatus, Holliston, MA, cat. no. AH 66-0008); silver chloride electrodes and electrode caps for Ussing chambers (Harvard Apparatus, cat. no. AH 66-0020 and AH 66-0023); forskolin (FSK; RBI, Westfield, MA, cat. no., F-105); 1-ethyl-2-benzimidazolone (1-EBIO; Tocris, Ellisville, MO, cat. No. 1041); DASU-01 can be obtained from the Bridges' Lab upon request. Low-noise high-bandwidth voltage-clamp and noise data acquisition hardware and software can be purchased from the van Driessche lab.[1]

3. Methods

3.1. T84 cell cultures and filter preparation

T84 cells are maintained in DMEM/F-12 (1:1) plus 5% FBS and 1% Pen-Strep culture medium. FBS is heat inactivated at 56 °C for 30 min. We typically add 100 ml FBS and 20 ml of Pen-Strep to 1900 ml DMEM and F12. The DMEM and F12 are prepared as described by the manufacturer. Cells are kept in an incubator at 37 °C in 5% CO_2 and passaged once per week. We have successfully passaged the cells for more than 1 year (i.e., over 52 passages) without any loss in transport phenotype. It is important not to let the cells become overly confluent before passage and thus avoid the need for excessive exposure to the trypsin–EDTA solution. To passage the cells, a 90% confluent T_{25} flask of cells is washed with 5 ml of PBS for 1 min. The 10× trypsin–EDTA is diluted 1:1 with PBS to yield a 5× solution. The 5 ml of PBS in the flask of cells is removed and 1 ml of trypsin–EDTA is added, washed across the cells and 0.5 ml is removed leaving just enough to cover the cells. The cells are incubated at 37 °C for 8–10 min until the cells detach from the flask. A 4-ml sample of media is added to the flask and the cells resuspended. About 1 ml of the resuspended cells is added to a T_{25} flask containing 4 ml of fresh media to passage the cells at a 1:4 split. To prepare a Transwell filter, 1 ml of the above cell suspension was diluted with 9 ml of fresh media and mixed well. Five drops of the cell suspension was added to the top of a Transwell filter. A 1.2-ml sample of fresh media was then added to the basolateral side. Both the flask and filters were fed the day after seeding and every other day thereafter. The apical surface of the Transwell is fed with 3 drops of medium. Filters are used 12–16 days after seeding, and we always fed the filters the night before using them. Grown in this manner, our T84 monolayers routinely have a transepithelial resistance of 1000–2000 Ω cm^2, a low basal I_{SC} of 1–3 μA/cm^2 and an I_{SC} response of 60–100 μA/cm^2 to maximal stimulatory concentrations of FSK (2 μM) and an I_{SC} response of 150–250 μA/cm^2 to FSK (2 μM) plus 1-EBIO (1 mM).

3.2. Ussing chamber solutions

The composition of the serosal buffer in millimolar is 120 NaCl; 25 $NaHCO_3$; 3.3 KH_2PO_4; 0.8 K_2HPO_4; 1.2 $MgCl_2$; 1.2 $CaCl_2$; 10 glucose. For the mucosal buffer, the 120 M NaCl is replaced with 120 mM Na gluconate and the $CaCl_2$ is increased to 4 mM. The pH of these solutions is 7.4 when gassed with a mixture of 95% O_2, 5% CO_2 at 37 °C. We typically prepared 500 ml of these buffers in a volumetric flask from 20× stock solutions of the above salts. When not in use, the buffers were kept at 4 °C and are gassed prior to each experiment. The solutions were warmed to 37 °C before use. This is important because cold solutions will stimulate Cl^- secretion in T84 cells. If the divalent salts have precipitated out of solution, usually because of the loss of CO_2 with storage or if there is any growth in the solution, the buffer is discarded. The osmolarity of the buffers is checked each time a new flask is

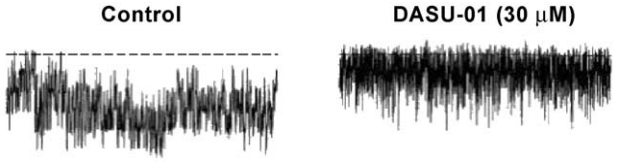

Fig. 2. Patch-clamp current traces of DASU-01 block of CFTR. Channel activity was measured in excised membrane patches from CFTR expressing L-cells in the presence of PKA and ATP (1 mM). The dashed line indicates the zero current level.

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prepared and should be approximately 290 mOsm. It is a good practice to measure the osmolarity each time a buffer is prepared since this is a good check that it has been prepared correctly.

3.3. Ussing chamber setup and mounting filters

The Costar vertical diffusion chamber modified to a Transwell filter is used to perform the noise studies. In their original design, the Costar chambers, now sold by Harvard Apparatus, were made to a Snapwell filter. In order to use a Transwell filter, the original Snapwell insert must be removed from the serosal side of the chamber, and a new insert that accommodates the Transwell filter must be made and inserted. In addition, the mucosal side of the chamber must be milled out to accommodate the Transwell filter. Our machine shop would be happy to modify the chamber for a modest fee.[2] In addition, one must purchase the electrode caps and electrodes to perform the noise studies. A detailed description of how to set up the Costar chamber and the care and handling of the electrodes can be found in Ref. [6]. For technical reasons, one must use the small surface area (0.33 cm^2) of the Transwell filters and not the larger surface area (1.1 cm^2) of the Snapwell filters. In addition, the voltage-measuring electrodes must be fashioned so that they are as close to the filter as possible, but not touching. In order to achieve this, the mucosal electrode must have a small bend near the tip so that it fits into the cup of the Transwell insert and thus can be placed close to the apical surface of the epithelium. Once all the electrodes are in place, the chamber is placed in a heating manifold and the electrodes are connected to the voltage clamp. The heating manifold we used for the noise studies was a custom aluminum jacket that surrounds the chamber and was mounted to an aluminum block that sits in a dri-bath-type heater (Fisher, cat. no. 11-716-68, Thermolyne, Dubuque, IA, number DB17615). The voltage clamp is a custom low-noise, high-bandwidth voltage clamp designed for the noise studies by Dr. van Driessche's laboratory [1,3]. The chamber and heater are set up inside a Faraday cage on a vibration-free air table. The cage and air table are essential so that background noise is minimal. The power cord to the heater is disconnected and placed in the cage whenever data are acquired for the current PDS. The heat capacity of the surrounding aluminum block is adequate enough to maintain the chamber temperature constant during the brief (5 min) period when data are acquired. The power cord is connected between data acquisition periods when additional drug is added or other experimental manipulations are performed. In addition, the gas supply is interrupted during the data acquisition phase. This sometimes causes a time-dependent decrease in the I_{SC} probably due to the development of an unstirred layer. If this occurs, we note the I_{SC} at the start and end of the data acquisition period for each PDS and use the mean I_{SC} in the calculations. The mucosal compartment is mixed before acquiring each PDS.

3.4. Performing a noise experiment on T84 cells

The current signal from the voltage clamp is connected to a pair of amplifiers and filters and then to a DSP board mounted in a PC.[3] Once a stable I_{SC} has been achieved, the acquisition of data to construct a PDS can be initiated. The desired fundamental frequency is selected, and the gain on the two amplifiers is adjusted to maximize the use of the full dynamic range of the DSP board. Depending on which fundamental frequency is selected (0.25, 0.5 or 1 Hz) each sweep of data will correspond to 4, 2 or 1 s of data, respectively, at a sample rate of 2048 samples per second. We typically acquire 20–30 sweeps of data for each PDS and three PDSs at each drug concentration at a fundamental frequency of 0.25 Hz. If a data sweep has too many data points beyond a preset threshold, the software automatically rejects that sweep of data. If too many sweeps of data are being rejected, it is then necessary to reduce the gain of the amplifier. If the sweep of data is accepted, a fast Fourier transform is performed, and the PDSs are averaged and displayed on the video monitor. After the preselected number of sweeps of data are acquired, the average PDS is saved, and it is then possible to begin acquiring additional data. One may also elect to fit the average PDS to the equations defining $1/f$ noise plus one or two Lorentzian functions (see below). The average PDS results and the fit with the estimated values of the parameters may then be saved. Thus, using this very user-friendly hardware and software, it is possible to perform a kinetic analysis as the experiment is being performed.

Consistent with the low basal I_{SC} of unstimulated cells, T84 cells display a low amplitude of current noise that can be described as $1/f$ noise. Stimulation with FSK or FSK plus 1-EBIO increases the I_{SC} and the amplitude of the noise as shown in Fig. 3. In most spectra, the PDS do not reveal a Lorentzian function in stimulated cells, and in general, the noise can be described as $1/f$ noise. The addition of DASU-01 over a concentration range of 10–100 μM leads to the appearance of a readily observable Lorentzian in the PDS. The subsequent addition of bumetanide, an inhibitor of the basolateral membrane $Na^+–K^+–2Cl^-$ cotransporter, causes a decrease in the I_{SC} a decrease in the amplitude of the power to the prestimulated values and the DASU-01-induced Lorentzian is no longer discernable (Fig. 3). DASU-01 has no effect on the PDS in unstimulated cells. These results are consistent with the conclusion that the DASU-01-induced Lorentzian is due to the blockade of apical membrane Cl^- channels. Pilot experiments revealed that FSK stimulation

[2] Please contact the corresponding author for further information.

[3] The noise hardware and software were developed by Dr. van Driessche and are available for purchase (see footnote 1).

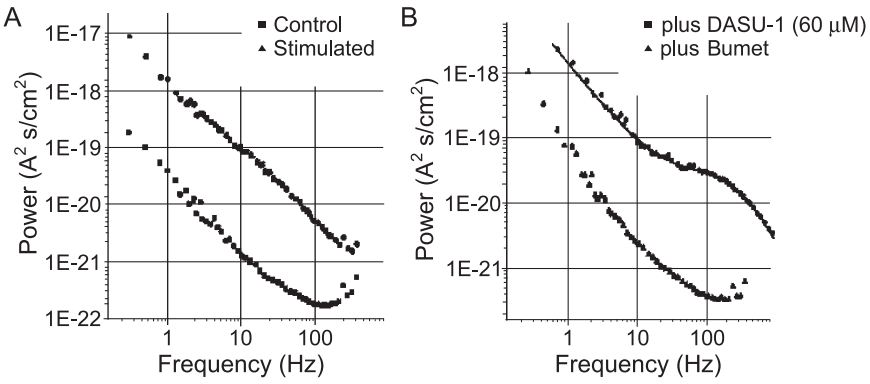


Fig. 3. Transepithelial power density spectra from T84 monolayers. Shown are the PDS for (A) control and stimulated conditions and (B) plus DASU-01 and bumetanide conditions corresponding to the experiment shown in Fig. 4. The solid line for the DASU-01 PDS is the fit to $1/f$ plus a Lorentzian component obtained by nonlinear regression.

alone was inadequate to obtain a DASU-01-induced Lorentzian in the PDS. Rather, it proved necessary to stimulate the cells with both FSK and 1-EBIO. 1-EBIO is an activator of basolateral membrane potassium (K^+) channels and is expected to increase the driving force for Cl^- exit across the apical membrane. In addition, it was necessary to have a Cl^--free mucosal solution to obtain a DASU-01-induced Lorentzian in the PDS. We interpret these results to suggest that the single-channel amplitude of CFTR in FSK and FSK plus 1-EBIO cells as being too low to be detected without also imposing a Cl^- gradient. Even under these conditions, the i of the DASU-01-sensitive channel was only 0.1 pA (see below). An alternative to the use of 1-EBIO is to permeabilize the basolateral membrane with α toxin and use the above Cl^- gradient with ATP and cAMP in the basolateral solution. Similar channel properties have been obtained using this alternative approach.

To perform an experiment, we first stimulate the T84 cells with FSK (2 μM) and 1-EBIO (1 mM) and allow the I_{SC} to become stable. DASU-01 (30 μM) is then added to both the serosal and mucosal solutions. Once the I_{SC} has reached a new plateau, three averaged PDSs of 30 sweeps each are obtained as described above. The DASU-01 concentration is then increased to 60 μM. While the I_{SC} is decreasing to a new plateau, the PDSs collected at the 30 μM concentration are fit and the values for the parameters (see below) are recorded. Three spectra are then obtained at 60 μM and the process repeated at 90 μM.

4. Data analysis

Fig. 4 shows a plot of $2\Pi f_c$ vs. the DASU-01 concentration (so called $2\Pi f_c$ plots or corner frequency plots) obtained from a typical experiment. f_c is the corner frequency of the fitted Lorentzian function (see below) at each blocker concentration. An open channel blocker is expected to yield a linear function such that

$$2\Pi f_c = k_{on}[B] + k_{off} \quad (1)$$

where k_{on} is the blocker-on rate and k_{off} is the blocker-off rate and [B] is the blocker concentration. Thus, the slope of the $2\Pi f_c$ plot is the k_{on} and the y-intercept is the k_{off}. As one can see from Fig. 4, DASU-01 block of FSK plus 1-EBIO stimulated T84 cells is well described by a linear function. From the estimated k_{on} and k_{off} one can calculate the equilibrium binding constant (K_D) as k_{off}/k_{on}. Together with the plateau value (S_o) of the fitted Lorentzian function at each concentration and the value of the I_{SC} one can calculate the channel properties as discussed below.

The short-circuit current (I_{SC}) across a Cl^- secreting epithelium can be written as

$$I_{SC} = iNP_o \quad (2)$$

where i is the single-channel amplitude of the Cl^- channel, N is the number of active channels, and P_o is the open probability of the channel. For each type of Cl^- channel that

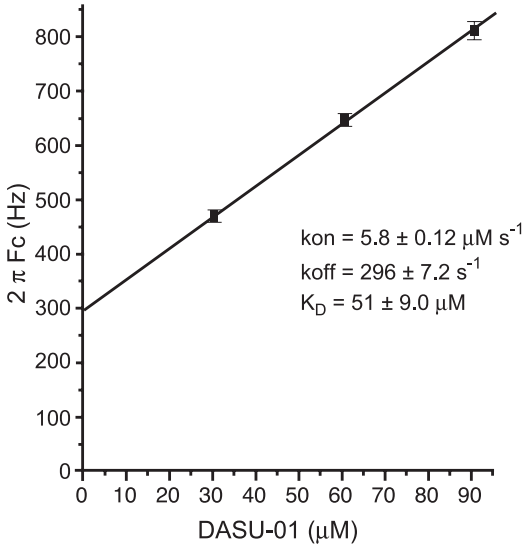


Fig. 4. Corner frequency plot for DASU-01 block of Cl^- secretion in T84 cells. Values are the mean ± S.E.M. for $n = 7$ monolayers. Experiments were performed as shown in Fig. 3.

contributes to the I_{SC}, an additional iNP_o term must be added to Eq. (2). Studies with the DASU-01 indicate 87% of the FSK plus 1-EBIO stimulated I_{SC} in T84 cells is mediated by a DASU-01 sensitive channel. The estimates of the DASU-01 k_{on}, k_{off} and K_D derived from the transepithelial noise studies are in excellent agreement with the values obtained in excised membrane patch-clamp studies of CFTR (data not shown). These results lend strong support to the conclusion that the DASU-01-induced transepithelial noise is the result of CFTR-mediated Cl^- secretion. Whether CFTR, in a DASU-01 insensitive conformation, mediates the remaining 13% of the I_{SC} or another DASU-01 insensitive channel is responsible for the remaining 13% will require additional investigation.

Current PDS are fit by nonlinear regression to one or more Lorentzian functions plus $1/f$ noise as given by Eq. (3).

$$S(f) = \frac{S_o}{1 + (f/f_c)^2} + \frac{S_1}{f^\alpha} \quad (3)$$

Where $S(f)$ is the power spectral density function, S_o is the plateau value of the Lorentzian component, f_C the corner frequency of the Lorentzian component, α is the negative slope of the $1/f$ noise and S_1 the power of the $1/f$ noise at 1 Hz. So far, we have observed only a single blocker induced Lorentzian in the PDSs from Cl^- secreting monolayers. However, under different experimental conditions, it is possible one might observe additional Lorentzian components arising from the spontaneous gating of the channels or alternative states of blockade. The additive nature of the noise signal permits one to include additional Lorentzian components in the fitting routine should they be observed under the different experimental conditions.

The single-channel amplitude (i) is calculated from the values S_o, f_c, k_{on} and I_{SC} at each blocker concentration according to Eq. (4):

$$i = \frac{S_o(2\pi f_c)^2}{4 I_{sc} k_{on}[B]} \quad (4)$$

When T84 cells were stimulated with FSK plus 1-EBIO i was 0.095 ± 0.011 pA and was constant over a DASU-01 concentration range of 10–90 μM.

The number of open channels (N_o) is given by Eq. (5):

$$N_o = I_{SC}/i \quad (5)$$

N_o, in the FSK plus 1-EBIO stimulated T84 cells, was 2.8×10^9 channels/cm^2 or 2800 channels/cell at zero blocker concentration.

The number of channels in the open (N_o) plus blocked (N_b) or N_{o+b} states at each blocker concentration is given by Eq. (6):

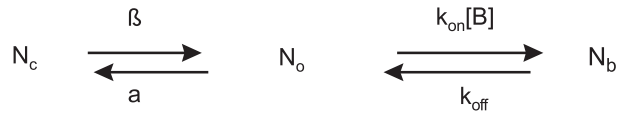
$$N_{o+b} = N_o(1 + [B]/K_D) \text{ where } K_D = k_{off}/k_{on} \quad (6)$$

It is important to note that the estimates of i, N_o and N_{o+b} do not assume any kinetic model for the spontaneous gating of the channel. Instead, the estimates rely on the blocker interacting with only the open state of the channel. The linear corner frequency plots for both the transepithelial noise and patch-clamp studies support the notion that DASU-01 is an open-channel blocker. In addition, the decrease in P_o caused by DASU-01 is fully accounted for by open-channel blockade. An open-channel block mechanism also predicts the recruitment of channels, by mass action, to the blocked state and will be reflected as an increase in N_{o+b} and a decrease in N_o as the blocker concentration is increased. These are indeed the results we have obtained with DASU-01 blockade of transepithelial Cl^- secretion. So far, there is no evidence that DASU-01 interacts with a closed state of CFTR. However, it is possible that under different experimental conditions this may occur and one should be alert to this possibility.

Given that a blocker interacts with only the open state of a channel in a pseudo-first-order reaction, one can obtain estimates of P_o by comparing the K_D, obtained from the k_{on} and k_{off} values, and the K_i from the inhibition in I_{SC}. The K_i for DASU-01 inhibition of the I_{SC} in T84 cells was 118 μM. Thus, if the channel has a P_o of 1.0, it will always be available for blockade and the K_D will equal the K_i. If P_o is some value less than 1.0, say 0.5, then the probability of the blocker encountering an open channel is reduced to 0.5 and the ratio of K_D to K_i will be 0.5. The difference in the K_D and K_i arises because there are two reaction mechanisms competing for the open state of the channel (i.e., closed and blocked). Actually, there can be any number of closed states from the open state or closed states connected to additional closed states and the ratio of K_D to K_i will still provide an estimate of P_o as long as the blocker displays a pseudo-first-order reaction with the open state of the channel. In other words, the estimate of P_o obtained by this method does not depend on a kinetic model for the spontaneous gating of the channel but only on the mechanism of action of the blocker. Using the ratio of the K_D (51 μM) to K_i (118 μM), the P_o of the DASU-01 sensitive channel in the FSK plus 1-EBIO stimulated T84 cells was 0.43 ± 0.025.

But what happens if the blocker does interact with a closed state of the channel in addition to the open state? If this were the case, then the ratio of K_D to K_i would give a false high estimate of P_o. Although, as indicated above, we have no evidence that DASU-01 interacts with a closed state of CFTR, it is possible that one might encounter, under different experimental conditions, a DASU-01-sensitive closed state. If this was to occur, one would expect to observe a new Lorentzian component in the PDS with a corner frequency equivalent to this new set of transitions (closed to blocked) and one should be alert to this possibility.

In addition to using the ratio of K_D to K_i to estimate P_o, one may also use methods that assume a kinetic model for channel gating. Helman and Baxendale [7] have described the derivations for several multi-state kinetic models and the expected experimental outcomes for an open or closed channel blocker. We have found that the simplest of these



Scheme 1.

models, the three state-closed–open-blocked model (Scheme 1), gave very similar estimates of P_o as those obtained from the ratio of K_D to K_i. According to this scheme, channels fluctuate between closed (N_C) open (N_o) and blocked (N_b) states (Scheme 1), and the open probability is given by Eq. (7):

$$P_o = \frac{1 - R}{R[B]/K_B} \tag{7}$$

Where R is the ratio of open channels in the presence of blocker to open channels in the absence of blocker. Open probability estimated by Eq. (7) was 0.38 ± 0.05 and 0.43 ± 0.025 by the K_D/K_i method for the experiments in Fig. 3, suggesting this simple model may be adequate to obtain estimates of P_o under these experimental conditions in T84 cells. However, it is important to recognize that the spontaneous gating of CFTR cannot be adequately described by a single closed–open set of transitions. Indeed, our own patch-clamp results argue for the requirement of at least three closed states to explain the ATP-dependent gating of CFTR and others have proposed even more complex models. However, because the blocker binds only to the open state, these three closed states are mathematically equivalent to a single competing closed state and the kinetic scheme can be represented as a three-state model (Scheme 1). Thus, while transepithelial noise is useful to obtain estimates of a channel's P_o under various experimental conditions, it is not appropriate to deduce a kinetic model for the gating of a channel.

5. Summary

The hardware, software, reagents and experimental protocol are now available to do transepithelial noise analysis of CFTR-mediated Cl^- secretion in T84 cells. Using these methods, we have observed a single-channel amplitude of approximately 0.1 pA, an open-channel density of 2800 channels per cell and an open probability of 0.4 in FSK plus 1-EBIO stimulated cells. Based on these values, there are approximately 7000 active channels (2800/0.4) per cell in the apical membrane of the maximally stimulated T84 cells, i.e., there are 7000 channels per cell fluctuating between the open and closed states in stimulated cells. There may also be many inactive channels in the apical membrane or inside the cell that would further increase the value of 7000, but these channels do not contribute to the secretion of Cl^- and go undetected in the noise studies. One can also estimate the apical membrane resistance from the number of open channels of 2.8×10^9 channels/cm^2 and the single-channel conductance of 10 pS to be approximately 35 Ω cm^2. Impedance analysis experiments yield an apical membrane resistance of approximately 40 Ω cm^2 in excellent agreement with the noise studies. Thus, the apical membrane of stimulated T84 cells has a very high conductance (low resistance) for Cl^-. To account for an I_{SC} of 200 μA/cm^2 (Cl^- current), a driving force of only 7 mV is required. The transepithelial noise analysis estimate of the P_o of approximately 0.4 is in excellent agreement with the patch-clamp observations of our own and that of several other labs. Thus, under physiological conditions in an intact monolayer and at maximal stimulatory concentrations of secretory agonists, one does not observe the high P_o (1.0) observed in some patch-clamp studies. Therefore, experiments using transepithelial noise can now be performed to investigate how the density and P_o of CFTR are regulated in an intact epithelium.

Acknowledgements

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